

**TITLE OF THE INVENTION**

ATP-DIPHOSPHOHYDROLASES, PROCESS OF PURIFICATION  
THEREOF AND PROCESS OF PRODUCING THEREOF BY RECOMBINANT  
TECHNOLOGY.

5 **FIELD OF THE INVENTION**

10 The present invention relates to a process of  
purification to homogeneity of ATP-diphosphohydrolases  
involved in numerous nucleotide and nucleoside receptor-  
mediated physiological functions, namely platelet  
aggregation, vascular tone, secretory, inflammatory and  
excretory functions and neurotransmission. These  
enzymes, which have been particularly obtained from  
bovine aorta and pig pancreas have been purified and  
their catalytic unit identified. The partial amino acid  
15 sequences of each ATPDase show a high degree of homology  
with a lymphoid cell activation system named CD39.

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## BACKGROUND OF THE INVENTION

ATP-diphosphohydrolases (ATPDases) or apyrases (EC 3.6.1.5) have been found in plants, invertebrates and vertebrates. The enzyme catalyses the sequential hydrolysis of the  $\gamma$ - and  $\beta$ -phosphate residues of triphospho- and diphosphonucleosides. These enzymes are generally activated in the presence of divalent cations  $\text{Ca}^{+2}$  or  $\text{Mg}^{+2}$  and inhibited by sodium azide. In plants, the enzymes are found in the cytoplasm, in soluble or membrane-associated forms, and are generally more active at acid pH. Their precise function is not known, but there is some evidence that they are involved in the synthesis of carbohydrates. In invertebrates, the enzymes are more active at neutral or alkaline pH. Found mainly in saliva and in salivary glands of hematophagous insects, an antihemostatic role has been demonstrated. In vertebrates, a limited number of studies have already defined a diversity of ATPDases. The catalytic site of these enzymes is generally exposed to extracytoplasmic spaces (ectoenzymes). By their

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location and kinetic properties, these different types  
of ATPDases could influence the main systems of the  
organism, namely vascular and nervous systems. Their  
specific role in these systems is determined by the  
presence of purine and pyrimidine receptors which react  
with triphosphonucleosides and their derivatives at the  
surface of numerous cell types.

Presence of both ectoATPase and ectoADPase  
activities in the vascular system has been known for  
many years, and up until the work of Yagi et al. (1989),  
they were attributed to two distinct enzymes. The  
latter purified these activities and showed that in  
bovine aorta, a single enzyme was responsible for the  
sequential hydrolysis of ATP and ADP. A mammalian  
ATPDase had been first described in the pancreas (Lebel  
et al., 1980) and was further reported in several other  
tissues. Yagi et al. (1989) proposed that the enzyme  
from aorta was similar to the previously reported  
mammalian ATPDase from pancreas and that it was  
associated with the intima of bovine aorta.

Purification to homogeneity was demonstrated by SDS-  
polyacrylamide gel electrophoresis (PAGE) and silver  
staining. The apparent molecular weight of the pure  
enzyme was estimated at 110 KDa. The existence of the  
5 ATPDase in the bovine aorta was corroborated by Côté et  
al. (1991) who, by showing that identical heat and  
irradiation-inactivation curves with ATP and ADP as  
substrates, assigned to the same catalytic site the  
ATPase and ADPase activities. A comparison of the  
10 biochemical properties led Côté et al. *supra* to propose  
that the bovine aorta enzyme was different from the  
pancreas ATPDase. Indeed, the enzymes have different  
native molecular weights, optimum pH and sensitivities  
to inhibitors. They proposed to identify pancreas  
15 enzyme as type I and the aorta enzyme as type II. In  
the bovine aorta, the enzyme was found to be associated  
with smooth muscle cells and endothelial cells and could  
inhibit ADP-induced platelet aggregation. Côté et al.  
(1991) further showed that concurrent addition of  
20 ATPDase and ATP to platelet-rich plasma resulted in an

immediate dose -dependent platelet aggregation caused by  
the accumulation of ADP, followed by a slow  
desaggregation attributable to its hydrolysis to AMP.  
In the absence of ATPDase, ATP did not induce any  
5 aggregation while ADP initiate an irreversible  
aggregation which extent is limited by the ADPase  
activity of the enzyme. ATPDase also attenuated the  
aggregation elicited by thrombin and collagen but not by  
PAF (Platelet Activating Factor), the first two agonists  
10 having an effect mediated by platelet ADP release. It  
was therefore suggested that ATPDase had a dual role in  
regulating platelet activation. By converting ATP  
released from damaged vessel cells into ADP, the enzyme  
induced platelet aggregation at the sites of vascular  
15 injury. By converting ADP released from aggregated  
platelets and/or from hemolyzed red blood cells to AMP,  
the ATPDase could inhibit or reverse platelet  
activation, and consequently limit the growth of  
platelet thrombus at the site of injury. In their  
20 attempt to further characterize the aorta ATPDase, the

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present inventors have developed a new process for  
producing highly purified ATPDases. They have  
established a procedure by which its specific activity  
can be increased over and above the activity of a crude  
5 cell preparation by more than 10000-fold. They also  
discover that the purified enzyme (the catalytic unit)  
had a molecular weight different from the one previously  
reported for the native form of the enzyme (190 KD by  
using the irradiation technique), suggesting that the  
10 enzyme may exist in a multimeric form in its native  
state. Partial amino acid sequences of both bovine  
aorta and porcine pancreatic ATPases have been obtained.

In a completely different field, Maliszewski et al.  
(1994) have published the sequence of a human lymphoid  
15 cell activation antigen designated CD39. Another group  
(Christoforidis et al. 1995) described the purification  
of a human placenta ATPDase of a molecular weight of 82  
KDa. Its partial amino acid sequence shows a high  
degree of homology with CD39.

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When the above mentioned partial amino acid sequences were entered in GenBank for verifying the presence of any homologous sequence, complete homology was surprisingly found for some of these fragments with the CD39 gene product. The complete sequences of the ATPDases remain to be obtained. Assuming that CD39 is an up to date unknown ATPDase, a process for producing ATPDases by recombinant technology is now possible, and CD39 can now be used to reduce platelet aggregation and thrombogenicity.

#### STATEMENT OF THE INVENTION

It is an object of the present invention to provide two ATPDases isolated from bovine aorta and porcine pancreas, which enzymes have a molecular weight for their catalytic unit of about 78 and 54 Kilodaltons, respectively. A novel process for obtaining a highly purified ATPDase is also an object of the present invention. This process has been successfully applied to the purification of both the pancreatic and the aorta

enzymes and is deemed to work in the purification of any ATPDase. For both sources of enzymes, the process allows the specific activity of the enzyme to be increased by at least 300 fold when compared to the activity retrieved in the microsomal fraction of these cells as previously reported for an aortic and pancreatic proteins of a native molecular weight of about 190 and 130 KDa, respectively.

The two ATPDases purified to homogeneity were partially sequenced. These sequences have shown striking similarities with a human lymphoid cell activation antigen named CD39 (Maliszewski et al., 1994). Since the molecular weight of CD39 and its glycosylation rate appears to define a human counterpart for the present bovine aortic ATPDase, it is the first time that a sequence is assigned to an ATPDase. A process of producing an ATPDase by recombinant technology is now possible using a host cell expressing the CD39 human protein, its homologous sequences in

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bovine and porcine species, and variants and parts thereof.

The present invention also relates to the use of CD39 and of the above bovine and porcine homologous proteins for reducing platelet aggregation and thrombogenicity.

#### DESCRIPTION OF THE PRESENT INVENTION

The research team to which the present inventors belong has already characterized the pig pancreatic ATPDase, and the latter reassessed the properties of the bovine aorta enzyme. They confirmed that the aorta ATPDase was different from its pancreatic counterpart. They have found previously (Côté et al., 1992) that the aorta enzyme (isolated from a microsomal fraction of the cells) had a molecular weight of about 190 kDa in its native state. In their work for extensively purify this enzyme, they found that the highly purified enzyme had a molecular weight on SDS-PAGE of about 78 kDa. Yagi et al. (1989) have already shown that an ATPDase purified

to homogeneity had a molecular weight of 110 KDa. After  
purifying the enzyme by the present method, the 110 kDa  
band was indeed absent from SDS-PAGE. A unique band  
migrating of an estimated weight of 78 KDa was rather  
revealed. The confirmation of the identity of the  
purified enzyme was achieved by binding FSBA, an ATP  
analog binding the enzyme, to the separated and blotted  
enzyme. The use of anti-FSBA antibodies revealed the  
presence of the bound enzyme and this binding was  
inhibited with ATP and ADP. The same procedure was  
applied to confirm the identification of the pancreas  
ATPDase Type I.

The present process allows the purification of  
ATPDases to a very high level. In the aorta, the  
purified enzyme has a specific activity which is  
increased by at least 300 fold compared with the  
specific activity of microsomal fraction (already  
enriched by about 30 fold from the crude cell  
preparation).

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The bovine aorta and porcine pancreatic ATPDases have been partially sequenced, and the sequences have been found to be highly homologous to a human lymphoid cell activation antigen designated CD39 (Maliszewski et al., *op. cit.*). The complete sequences of the ATPDases types I and II have not been obtained yet. If one assumes that CD39 gene product is an ATPDase type II, the present invention therefore contemplates the use of CD39 in the reduction of platelet aggregation and of thrombogenicity, as well as a process of making ATPDases using the CD39 sequence, variants or parts thereof (recombinant technology).

The present invention will be described hereinbelow with reference to the following Examples and Figures which purpose is to illustrate rather than to limit the scope of the present invention.

# BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the protein composition of the bovine aorta ATPDase (type II) at the different purification steps as determined by SDS-PAGE.

5 Electrophoresis was run in a 7-12.5% polyacrylamide gel. Proteins were stained with Coomassie Blue or silver nitrate dye. MW standards: 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 KDa; particulate fraction (part. fract.), 100 µg; DEAE-agarose fraction, 35 µg; Affi-Gel  
10 blue fraction, 20 µg; the lower band of activity was cut out from the non-denaturing gel (N.D. gel); sample buffer alone (Control).

Figure 2 illustrates a Western blot of FBSA labelled protein (ATPDase type II) isolated from Affi-Gel blue  
15 column. Labelled proteins were separated on a 8-13.5% gradient gel by SDS-PAGE, transferred to Immobilon-P membrane, incubated with a rabbit antibody anti-FBSA (1:10,000) and detected by a secondary antibody conjugated to alkaline phosphatase (1:6,000). Twenty µg  
20 of protein from Affi-Gel blue column fraction was used

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for the assays: incubation with FBSA (FBSA); incubation with FBSA with competing Ca-ATP (FBSA+ ATP); incubation without FBSA (no FBSA). MW standards are the same as in Figure 1.

5 **Figure 3** illustrates the SDS-PAGE protein patterns at the different steps of the purification procedure and after N-glycosidase F digestion of the Affi-Gel blue fraction. Protein samples were fractionated on a 8-13.5% polyacrylamide gradient. A) One unit of N-glycosidase F (silver nitrate stain); B) Six  $\mu$ g from the Affi-Gel blue fraction incubated for 12 h without N-glycosidase F (silver nitrate stain); C) Idem as B with 1 unit of N-glycosidase F (silver nitrate stain); A') Same as A (Coomassie blue stain); B') Same as B (Coomassie blue stain); C') Same as C (Coomassie blue stain); D) MW standards: 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa (Coomassie blue stain), E) ZGM (zymogen granule membrane), 60  $\mu$ g (Coomassie blue stain); F) Active fraction from DEAE-agarose column, 25  $\mu$ g (Coomassie blue stain); G) Active fraction from Affi-Gel blue column, 6

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µg (Coomassie blue stain); G') Same as G (silver nitrate overstain); H) Activity band located after PAGE under non-denaturing conditions (silver nitrate overstain); I) Control, band located just above the activity band after PAGE under non-denaturing conditions (silver nitrate overstain).

**Figure 4** shows a Western blot of FSBA labelled samples of the pancreatic enzyme type I fraction. Labelled sample were loaded on a 7-12% polyacrylamide SDS-gel, transferred to Immobilon-P membrane, incubated with the rabbit antibody anti-FSBA and detected by a secondary antibody conjugated to alkaline phosphatase. Six µg of Affi-Gel blue column were used in lanes B), C) and D). A) MW standards: 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 kDa; B) FSBA; C) FSBA + competing ADP; D) No labelling.

**Figure 5** shows a Western blot of human endothelial cell extracts labelled with an antibody directed against a fragment common to ATPDase type I and CD39. The ATPDase type II (78KDa) is clearly detected as well as low amounts of ATPDase type I (54KDa).

**Example 1**

PURIFICATION OF THE ATPDase type II

a) Isolation of the particulate (microsomal) fraction from the bovine aorta:

5           Bovine aorta, obtained from a local slaughterhouse,  
were kept on ice and processed within one hour after the  
death of the animals. All steps were carried out at  
4°C. The inner layer was stripped out manually, passed  
through a meat grinder, and homogenized (10%) with a  
10   Polytron™ in the following solution: 95 mM NaCl,  
Soybean Trypsin Inhibitor (20 µg/mL), 0.1 mM Phenyl-  
methyl-sulphonyl-fluoride (PMSF) and 45 mM Tris-HCl pH  
7.6. After filtering with cheesecloth, the homogenate  
was centrifuged at 600 X g for 15 minutes with a Beckman  
15   JA-14 centrifuge at 2100 RPM. The supernatant was  
recovered and centrifuged at 22,000 X g for 90 minutes  
with the same centrifuge at 12,000 RPM. The resulting  
pellet was suspended in 0.1 mM PMSF and 1 mM NaHCO<sub>3</sub> pH  
10.0 with a Potter Elvehjem™ homogenizer at a dilution  
20   of 3 to 6 mg of protein per mL. The suspension was

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loaded on a 40% sucrose cushion and centrifuged at 100,000 X g for 140 minutes with a SW 28 Beckman rotor. The enzyme was recovered on the cushion and kept at 4°C overnight. This membrane preparation was then suspended in 12 volumes of 0.1 mM PMSF and 1 mM NaHCO<sub>3</sub> pH 10.0 and centrifuged at 240,000 X g for 45 minutes in a SW 50.2 Beckman rotor. The pellet was rinsed twice: once with 0.1 mM PMSF and 30 mM Tris-HCl pH 8.0 and once with 2 mM EDTA and 30 mM Tris-HCl pH 8.0. The final pellet was suspended in 7.5% glycerin and 5 mM Tris-HCl pH 8.0 at a concentration > 1 mg of protein per mL and frozen at -20°C, or directly solubilized. At this stage, the specific activity of the ATPDase was enriched by about 33 fold.

b) Solubilization and column chromatographies:

The particulate fraction (pf) was solubilized with 0.3% Triton X-100™ and 30 mM Tris-HCl pH 8.0 at a concentration of 1 mg/mL protein and centrifuged at 100,000 X g for 1 hour in a SW 50.2 Beckman rotor. All further steps involving a detergent are practised with

Triton X-100, but any similar detergent (a non-ionic detergent) may be used for achieving the purpose of this invention. The supernatant was loaded on an ion exchange column, preferably containing diethylaminoethyl (DEAE), like DEAE-Bio Gel A Agarose™, preequilibrated with 0.1% Triton X-100™, 7.5% glycerin and 10 mM Tris-HCl pH 8.0. The protein was eluted in the same buffer by a NaCl gradient (0.03 to 0.12 M), followed by a 0.1% Triton X-100™ and 2 M NaCl wash. Active fractions were pooled in 0.1X buffer E (5X buffer E: 0.5% Triton X-100™, 960 mM glycine, 125 mM Tris-HCl pH 7.0) and electrodialed in 15 mL cuvettes by an ISCO™ electro-eluter according to the following technique: 1X buffer E was loaded in the apparatus and a 15 mA current was applied per cuvette. The 1X buffer E was changed 4 times at 50 minute intervals. The dialysate was equilibrated at pH 5.9 with 200 mM histidine adjusted to pH 4.0 with HCl (about 20 mM final) and loaded on an Affi-Gel™ blue column preequilibrated with 0.07% Triton X-100™, 7.5% glycerin, 30 mM histidine and 30 mM Tris-HCl pH 5.9. Proteins

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were eluted by a linear gradient from 100% buffer A to 100% buffer B (buffer A (80 ml): 0.07% Triton X-100™, 7.5% glycerin and 10 mM Tris-HCl pH 6.5; buffer B (80 ml): 1M NaCl, 0.07% Triton X-100™, 7.5% glycerin and 10 mM Tris-HCl pH 7.5), followed by a 1M NaCl, 0.1% Triton X-100™, 100 mM Tris-HCl pH 8.5 wash. The active fraction was dialysed against 0.05% Triton X-100™, 1 mM Tris-HCl pH 8.0, concentrated on a 1 ml DEAE-agarose column as described above, eluted in 0.4 M NaCl, 0.07% Triton X-100™, 10 mM Tris-HCl pH 8.0 and dialysed against distilled water.

c) Separation by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions:

15        This type of gel allows for separating proteins upon their molecular weight and electrical charge while preserving their activity in such a way that this activity can be measured after migration. Two polyacrylamide preparations were poured between two glass plates to form a gradient and polymerized. The 4%

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acrylamide solution was composed of: 4.5 mL of separating buffer (Tris 1.5 M pH 8.8+ 0.4% Triton X-100™), 2.5 mL acrylamide 30%, 180 µL Na deoxycholate 10%, water up to 18 mL, 60 µL APS 10% and 7 µL TEMED.

5 The 7.5% acrylamide solution was composed of the same ingredients except for the volume of acrylamide: 4.5 mL.

A stacking gel was extemporaneously prepared and poured at the top of the separating gel, the stacking gel was composed of: 2.5 mL of stacking buffer (Tris-base 0.5 M pH 6.8), 6.1 mL of water, 1.34 acrylamide 30%, 0.1 mL Na deoxycholate 10%, 0.1 mL Triton X-100™, 50 µL APS 10% and 10 µL TEMED. Wells are formed in this layer during polymerization. Two volumes of the sample obtained after DEAE-agarose or Affigel Blue columns were added to one volume of sample buffer of the following composition to obtain about 100 µg proteins: 0.07% (v/v) Triton X-100™, 1.5% (w/v) Na deoxycholate, 10% glycerol, 65 mM Tris-base and 0.005% bromophenol blue. The suspended sample was allowed to stand 10 minutes on ice and centrifuged. The supernatant was loaded on gel. The

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proteins were migrated at 4°C at a 20 mAmp power in reservoir buffer (0.1% Triton X-100, 0.1% sodium deoxycholate, 192 mM glycine and 25 mM Tris pH 8.3). For revealing activity in the separated bands, the latter

5 were placed in a dosage buffer (Tris-base 66.7 mM, imidazole 66.7 mM,  $\text{CaCl}_2$  10 mM, pH 7.5). After preliminary incubation for 30 minutes at 37°C, the substrate (ADP or ATP) 5 mM was added. After 2 to 10 minute incubation, a white calcium phosphate precipitate

10 significative of ATP diphosphohydrolase activity is formed. Three bands are seen for the aorta enzyme and one for the pancreas (these bands were all revealed on gel by silver overstaining). For further characterization, the most active band was loaded on an

15 SDS-PAGE according to Laemmli (1970) and a single band appeared on the gel after silver nitrate staining, which is indicative of an enzyme purification to homogeneity after the non-denaturing gel. Figure 1 shows the high sensitivity of detection conferred by the use of silver

20 staining compared to a conventional Coomassie blue

staining (see lanes 4 and 5). The active band purified from the gel has a molecular weight of 78 KDa when migrated on SDS-PAGE.

d) ATPDase assays during chromatographic steps:

5        Enzyme activity was determined at 37°C in the following incubation medium: 50 mM Tris-imidazole (pH 7.5), 8 mM CaCl<sub>2</sub> and 0.2 mM substrate (ATP or ADP). Phosphorus was measured by the malachite green method according to Baykov et al. (1988). One unit of enzyme  
10        corresponds to the liberation of 1 μmol of phosphate per minute per mg of protein at 37°C. Proteins were estimated by the technique of Bradford (1976).

The ATPDase activity retrieved in isolated fractions are summarized in the following Table:

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Table 1. ATPDase purification of the bovine aorta ATPDase type II.

Step	Total		Specific activity		Yield	Purification factor	Hydrolysis rate
	protein	activity	units	units/mg			
	mg	units	units/mg	%	-fold	ATP/ADP	
Particulate fraction (pf)	293	263	0.9	-	(33)*		1.5
pf + Triton X-100	293	117	0.4	100	1		1.4
100,000 g supernatant of	186	91.2	0.5	78	1.2		1.3
solubilized pf							
DEAE column	15.1	72.2	4.8	62	11.9		1.1
Affi-Gel blue column	2.76	57.8	21	49	53		1.1
Con A	0.61	33.5	55	29	138		1.1

Details on the purification and condition assays are described in the disclosure. A representative out of five complete purification procedures is shown with ADP as substrate. Determinations were routinely carried out in triplicate. \* The starting particulate fraction shows a 33 purification folds as compared to the homogenate (Côté 1991).

e) Confirmation of the identity of ATPDase:

5 The fraction eluted from Affi-gel was labelled with  
5'-p-fluorosulfonylbenzoyl adenosine (FSBA), a marker  
which forms covalent bonds with adenosine-binding  
proteins. FSBA blocked the enzyme activity and excess  
of ATP or of ADP prevents this effect. In addition,  
FSBA efficiently bound the purified enzyme, as monitored  
by a Western blot technique using an antibody directed  
to FSBA, which binding is prevented in the presence of  
10 ATP (see Figure 2) or ADP (data not shown).

15 The results obtained on SDS-PAGE shows that the  
enzyme was purified to homogeneity when using the  
successive steps of solubilization of the particulate  
fraction, first purification on an ion exchange column,  
second purification on an affinity column and third  
purification on non-denaturing electrophoretic  
conditions. The Affigel Blue column did not achieve  
purification to homogeneity but allowed a much higher  
recovery than the 5' AMP-Sepharose™ used by Yagi et al.  
20 (about 7 fold higher). Moreover, the use of the Affigel

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column and the non-denaturing gel allowed us to purify an enzyme that is different from the one disclosed by Yagi.

f) ATPDases are glycosylated proteins:

**Purification on Concanavalin A column:**

5 Further purification of the Affi-Gel blue fraction of  
aorta enzyme was also obtained with Con A agarose  
column. Briefly, Con A (4 ml beads) and the protein  
sample from the Affi-Gel blue column were  
10 preequilibrated with 0.05 % Triton X-100, 100 mM NaCl,  
1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MnCl}_2$  and 20 mM PIPES, pH 6.8, at room  
temperature. The protein sample was passed through the  
column at a flow rate of 3 ml/h, 40 ml of the  
preequilibration buffer was then added to wash the  
15 unbound materials at a flow rate of 10 ml/h. The  
activity was eluted with 20 ml of 0.5 M  
Me- $\alpha$ -D-mannopyranoside diluted in the preequilibration  
buffer. The purified sample was dialysed and  
concentrated on a mini-DEAE column as described above.

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Precipitation of ATPDase activity with  
lectin-agarose:

Four lectins conjugated to agarose were tried: Con  
A, WGA, Soybean agglutinin and UEA. Experiments were  
5 carried out at room temperature for Con A, and at 4°C  
for the other agglutinins. One hundred  $\mu$ l of each 50%  
slurry were put in a microcentrifuge tube and washed 4  
times with buffer A: 0.05% Triton X-100, 100 mM NaCl and  
20 mM PIPES pH 6.8. In the case of Con A, 1 mM  $\text{CaCl}_2$  and  
10 1 mM  $\text{MnCl}_2$  were added to this buffer. Twenty  $\mu$ g of  
ATPDase purified from the Affi-Gel blue column,  
equilibrated in buffer A, were added to the  
lectin-agarose beads and rocked for 45 min, then  
centrifuged for 1 min. The supernatant was kept and the  
15 beads were washed 3 times with 1 ml buffer A. Protein  
bound to the lectins was eluted with 150  $\mu$ l of 500 mM of  
the appropriate sugar in buffer A, rocked for 30 min and  
centrifuged. The elution step was repeated once and the  
2 eluates were pooled. The sugar used to eluate proteins  
20 from Con A, WGA, Soybean and UEA were

Me- $\alpha$ -D-mannopyranoside, D-GlcNAc, D-GalNAc and L-Fuc  
respectively.

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Table 2. ATPase binding to lectins

Lectin- agarose	Fractions	Relative ADPase activity	Presence of the 78 kDa band on SDS-PAGE	Sugar specificity
Con A	Supernatant	5%	traces	Mannose,
	Bound	95%		Glucose
	Eluted	62%	+	
WGA	Supernatant	5%	traces	GlcNAc, NeuNAc,
	Bound	95%		Mannose structure §
	Eluted	69%	+	Sialic acid §
Soybean	Supernatant	100%	+	GalNAc
	Bound	0%		
	Eluted	0%	-	
UEA	Supernatant	100%		Fucose
	Bound	0%	+	
	Eluted	0%	-	

Twenty µg of ATPase fraction purified by Affi-Gel blue chromatography were incubated separately with four lectins conjugated to agarose, centrifuged, and the supernatants were collected. Lectins-agarose beads were then washed. Bound proteins were finally eluted with the appropriate sugar as described in the disclosure. This experiment has been done twice in triplicate and the mean is presented. In parallel, the supernatant and the eluted fraction were put on SDS-PAGE, stained with silver nitrate, and looked for the presence of the 78 kDa. The sugar specificity of each agglutinin is also presented.

§ Weak affinities

Only WGA bound the ATPDase type II as for Con A. ATPDase binding to these two lectins is indicative of a specificity for the sugars glucose and/or mannose and/or GlcNAc (Glucosamine-N-Acetyl) and/or NeuNAc (Neuraminic-N-Acetyl).

The deglycosylated form had a molecular weight of about 56 KDa, which suggests that about 5 to 11 glycosyl chains are present on the 78 KDa protein (assuming that a glycosyl group may have a molecular weight of 2 to 4 KDa).

#### Example 2

##### PURIFICATION OF THE ATPDase type I

The procedure described in Example 1 has been followed for purifying the pancreatic ATPDase type I enzyme, starting from the zymogen granule membrane of pig pancreas.

In deglycosylation experiments, the molecular weight of the catalytic unit has been shown to be shifted from 54 to 35 KDa. Therefore, the chemical

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procedure exemplified above is deemed to apply to the purification of ATPDases in general.

h) Level of enrichment:

5 The level of enrichment is determined from the data shown in Table 1 for aorta ATPDase type II and from the following Table 3 obtained for pancreatic ATPDase type I.

10 From the crude cell preparation to the Affigel Blue column, the enzymes of both pancreatic and aorta sources were purified to at least a 1600 fold level (see Tables 1 and 3. After the non-denaturing gel, the quantity of proteins falls under the detection level of the method used, which renders difficult the calculation of a specific activity. However, one can roughly estimate  
15 the process to reach about a 10 thousand fold purification, as judged by the density of the ATPDase reaction band relative to other proteins on the non-denaturing electrophoretic gel.

20 Referring to Table 1, the lectin-binding step is not considered properly as an essential step of the

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purification process. This step has been added to show that the aorta ATPDase is a glycoprotein which, when deglycosylated, shifts from a molecular weight of 78 KDa to a molecular of 56 KDa (representing the proteic backbone). Since the lectin-binding step does not achieve the obtention of a pure protein, the most convenient way to obtain a pure protein is to submit the crude cell preparation sequentially to the ion exchange chromatography, the Affigel Blue chromatography and to non-denaturing gel electrophoresis. The identity of the protein is then confirmed by ATP-labelling with FSBA.

### Example 3

#### Partial amino acid sequences

CNBr digests have been obtained from the purified bovine aorta and porcine pancreatic ATPDases. The sequences of the digests are as follows:

SEQ. ID.

NO. :

Giu. 1971

	5	10	3
5	.		

5 10 5

Tyr Pro Phe Asp Phe Gln Gly Ala Arg Ile  
5 10 6

Lys Ser Asp Thr Gln Glu Thr Tyr Gly Ala

Leu Asp Leu Gly Gly Ala Ser Thr Gln Val

15 20 7

When compared to the sequence which accession number is G2345 (CD39 gene product; Maliszewski et al. 1994), the above partial sequences show a very high degree of homology. The following differences are however found with the CD39 sequence:

In the porcine pancreatic enzyme, Gln<sup>202</sup> is changed to Lys, the Asn<sup>204</sup> is changed to Asp, Asn<sup>205</sup> is changed to Thr.

In the bovine aortic enzyme, Arg<sup>147</sup> is changed to Lys, Val<sup>148</sup> is changed to Ile, Asp<sup>150</sup> is changed to Ala, Gln<sup>153</sup> is changed to Ala, Arg<sup>154</sup> is changed to Ser, and Leu<sup>156</sup> is changed to Ile.

The human CD39 has a predicted molecular weight of 57 KDa, while the apparent molecular of this protein is 78KDa on SDS-PAGE.

Both ATPDases type I and II share a high degree of homology with CD39 for the compared sequenced fragments. CD39 appears to be a human enzyme corresponding to the bovine aortic ATPDase. It is worthwhile noting that the first N-terminal 200 amino acids of CD39 are absent from

the ATPDase type I (pancreatic enzyme). This suggests  
that the active site of ATPDases is located between the  
residues 200-510 of CD39 and that part of CD39 is  
sufficient to provide this activity. It is further  
5 worthwhile noting that exact correspondence between the  
two ATPDases of this invention and the already described  
ATPDases cannot be established. The human placenta  
ATPDase (Christoforidis et al. 1995) has a molecular  
weight of 82KDa while CD39 (also of human origin) has a  
10 molecular weight of 78KDa. Due to the differences found  
in diverse tissues of the same species, extrapolation  
cannot be done to the effect that the bovine aorta  
enzyme of this invention is one of the already described  
enzymes. The obtained partial amino acid sequences  
15 indeed already shown differences of sequences which may  
affect some of the physico-chemical properties of the  
claimed enzymes when compared to their human  
counterparts (some of the above-observed substitutions  
are not conservative ones; the net charge of the enzymes  
20 may not be the same and the substituted amino acids may

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change the behaviour of the enzymes (optimum pH, sensitivity towards inhibitors, etc ...).

Cross-reactivity between ATPDases I and II:

Antibodies were produced in rabbits against the following amino acid sequence which is common to ATPDase I and CD39:

SEQ. ID.

NO.:

Lys Ser Asp Thr Gln Glu Thr Tyr Gly Ala

5

10

Leu Asp Leu Gly Gly Ala

15

8

Figure 5 shows that these antibodies reacted positively with a 78KDa protein present in endothelial extracts of human sources. They also reacted with a protein of 78 KDa of a bovine aorta extract (data not shown). This is an indication that ATPDases I and II share homology of sequence, and that the latter comprises the peptidic sequence of SEQ. ID. No.: 8 or a variant thereof.

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A type I ATPDase appears to be present in low amounts in endothelial cells as shown by the detection of a faint band corresponding to this protein (54KDa) in Figure 5.

5      **CONCLUSIONS:**

-Considering that the ATPDase has an antihemostatic role in the saliva of blood-feeding insects and leeches (Rigbi et al., 1987);

10      -considering that Côté et al. (1992) have demonstrated bovine ATPDase type II has platelet anti-aggregant properties by converting ADP to AMP;

15      -considering the low  $K_m$  of the aorta type II enzyme ( $\mu M$ ), the optimum pH of catalysis pH 7.5-8.0, its localization at the surface of endothelial and smooth muscle cells of blood vessels (Côté et al., 1992);

-considering that the purified enzyme keeps its original characteristics;

it sounds predictable that the aorta enzyme produced in the present invention can be introduced in

09781796-021201

the circulatory system of mammals to reduce platelet aggregation and thrombogenicity.

Furthermore, considering that a crude microsomal bovine ATPDase type II fraction has been successfully conjugated to agarose and that the conjugate could reduce ADP induced platelet aggregation (Hirota et al., 1987);

-considering that a semi-purified plant ATPDase has been successfully coupled to the following matrices: CM-cellulose, copolymers of L-alanine and L-glutamic acid, polyaspartic acid, polygalacturonic acid, Elvacite 2008™ (methyl methacrylate) and ethylene-maleic acid copolymer (Patel et al., 1969);

we propose that the purified ATPDase type II can be coupled to artificial polymers/biomaterials to reduce thrombogenicity (platelet aggregation).

Therefore, pharmaceutical compositions for use in the reduction of platelet aggregation and thrombogenicity are under the scope of the invention. These compositions should contain, as an active

ingredient, the ATPDase type II of this invention  
combined to an acceptable carrier without excluding any  
form or formulation of such compositions. Finally,  
considering that the sequenced CD39 appears to  
5 correspond to a human counterpart of the bovine ATPDase  
type II enzyme of this invention, the use of CD39 or  
variants or a part thereof for reducing platelet  
aggregation and thrombogenicity is also part of this  
invention.

10 A new process for producing an ATPDase comprising  
the steps of:

- obtaining a host which comprises a nucleic acid  
encoding a protein having the amino acid sequence  
defined in SEQ. ID. NO.: 1, or a variant thereof, or a  
15 part thereof, said variant or part being capable of  
converting ATP to ADP and ADP to AMP;

- culturing said host in a culture medium  
supporting the growth of said host and the expression of  
said nucleic acid;

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- recovering the ATP diphosphohydrolase from the culture medium or from said host; and

- purifying the ATP diphosphohydrolase

5 is also part of the invention. Preferably the nucleic acid is the one defined in SEQ ID NO.: 2, or a part or a variant thereof, which part or variant is capable of producing an ATP diphosphohydrolase.

10 The present invention has been described hereinabove; it will become apparent to the skilled reader that variations could be brought thereto without departing from the teachings of the present disclosure. Such variations are under the scope of this invention.

09781796-021201

TABLE 3

ATPDase purification

Results of one out of three preparations is presented. Determinations were carried out in triplicate.

\* Laliberté et al. showed a 160 fold purification for the ZGM as compared to the homogenate using ADP as the substrate.

Steps	Total protein	Total activity	Specific activity (ATP)	Yield %	Purification factor	Hydrolysis rates ATP/ADP
	mg	units	units/mg	%	fold (160) *	
ZGM	20.0	60.8	3.0	-	1	1.3
ZGM + Triton X-100	20.0	40.6	2.0	100		1.3
100,000 g supernatant	17.6	37.0	2.1	91	1.1	1.3
of solubilized						
ZGM						
DEAE column	3.5	28.8	8.3	71	4.2	1.3
Affi-Gel blue column	0.31	13.8	45	34	23	1.3

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